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Original Article

A mechanical acupuncture instrument mitigates the endoplasmic reticulum stress and oxidative stress of ovariectomized rats

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ABSTRACT

Background: Acupuncture has become a common complementary and alternative treatment approach for anxiety and depression. However, there is little research on the detailed mechanism of acupuncture therapy relieving depression. Previously, 17β-estradiol (E2) was shown to prevent oxidative stress and endoplasmic reticulum (ER) stress in ovariectomized (OVX) rats. This study investigated whether stimulation of Sanyinjiao (SP6) using a mechanical acupuncture instrument can alleviate depression-like behavior caused by estrogen deficiency in OVX rats. Furthermore, we found that acupuncture reduced ER stress and oxidative stress-related proteins expression.

Methods: The OVX operation was performed on female SD rats that were separated into four groups: The E2 (2.5 μg/kg, i.p.) injection group (OVX + E2), the OVX group (OVX), and the OVX with acupuncture stimulation group (OVX + SP6). Non-acupoint stimulation group (OVX + NonAcu). The acupuncture point stimulation began three weeks after surgery. The depressive behavior was analyzed by the forced swim test and open field test. The 8-OHDG, BiP, Sigma receptor 1, pJNK, PDI, Ero1-Iα and Calnexin protein levels were evaluated by immunoreactivity in the amygdala.

Results: Acupuncture stimulation reduced depressive behavior and altered depression-related proteins. Stimulation of SP6 decreased the immobility time of the FST and altered the ER stress and oxidative stress marker proteins, such as 8-OHDG, BiP, pJNK, PDI, Ero1-Ia and Calnexin.

Conclusion: Our results indicated that acupuncture at SP6 showed a significant antidepressant-like effect on an OVX-induced depression rat model by mitigation of ER stress and oxidative stress in amygdala.

1. Introduction

Estrogen is the major female sex hormone and exerts effective neurotrophic and neuroprotective effects in the brain.[1–3] The possible neuroprotective effects of estrogen mechanisms have been proposed for the such as regulation of gene transcription, antioxidant effects and activation of membrane-associated intracellular signaling pathways.[2] According to recent studies, estrogen has been shown to exert neuroprotective effects in numerous neurodegenerative situations involved in depression.

The unfolded protein response (UPR) is a cellular stress response related to the endoplasmic reticulum (ER). The UPR is activated in response to accumulation of misfolded or unfolded proteins in the ER lumen. If UPR roles are not attained within a certain time span or the disruption is prolonged, the UPR promotes apoptosis. Sustained over-activation of the UPR has been implicated several neurodegenerative diseases, and inhibiting the UPR could be a treatment for these diseases. Notably, the UPR system plays an central role in major depressive disorder.[4] The generation of reactive oxygen species (ROS) and protein folding as a byproduct of protein oxidation in the ER are closely linked events. Both ER stress and oxidative stress, through ROS generation, may increase leakage of Ca^{2+} from the lumen of the ER. Increased Ca^{2+} expression is induced the various protein imbalances. It has been reported to modulate several behaviors, including learning and memory, anxiety, and depression.[5–7]

Many studies have shown that estradiol can relieve the protein imbalance caused by leakage of Ca^{2+}.[8,9] Treatment with 17β-estradiol inhibited ER stress-induced apoptosis of primary osteoblasts.[10] Additionally, 17β-estradiol prevented oxidative stress and decreased blood pressure in ovariectomized (OVX)

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rats.[11] Despite these findings, studies on ER stress and oxidative stress in estradiol-deficient menopausal depression are lacking.

The amygdala is an important brain region closely related with stress reactivity and vulnerability for depressive disorder. The amygdala metabolic activity was found to be correlated with negative mood. Estradiol modulates mood and cognition via interaction with estrogen receptors in key brain regions, such as the hippocampus, amygdala and prefrontal cortex. Despite the importance of the amygdala, ER stress and oxidative stress in this region have been poorly studied.

Acupuncture is a traditional complementary and alternative medicine approach that includes inserting needles into specific acupuncture points to restore proper energy flow inside the body.[12] Notably, one important effect of acupuncture is regulation of the ER stress response for apoptosis through the UPR and Ca^{2+} homeostasis.[13,14] The antioxidative effects of acupuncture stimulation are also an important mechanism of apoptotic defense. For example, the beneficial effects of acupuncture treatment in the 6-OHDA lesioned rat model and vascular dementia rat model might be mediated by regulation of the antioxidant defense system.[15,16] Additionally, limb ischemia–reperfusion research showed that electroacupuncture (EA) has neuroprotective possible mediated, at least in part, by decrease of oxidative stress and inhibition of microglial activation.[17] In addition, various studies have shown that acupuncture restores apoptosis in different disease conditions.[18–20] Many studies have indicated that acupuncture stimulation is effective for depression and that its underlying mechanism involves mitigating apoptosis.[21,22] Despite the various studies showing that acupuncture stimulation is effective for depression, the effects of antioxidant and ER stress-related depression have not been studied yet.

Based on the abovementioned studies, we hypothesized that acupuncture therapy would reduce depressive behavior and ER stress and have antioxidant effects. According to the resent researches, the acupuncture stimulation at the (Sanyinjiao) SP6 point improve of women's universal health. Recent studies have shown that acupuncture at SP6 significantly decreased depression behaviors in the OVX rat. For this reason, this study used the acupuncture point SP6 in the OVX rats.[23] Therefore, the present study was considered to examine the following: (1) whether mechanical acupuncture of acupuncture point SP6 would decrease depression-like behavior, (2) whether acupuncture stimulation would reduce ER stress and oxidative stress in various brain regions, and (3) whether acupuncture stimulation would reduce the various proteins related to ER stress, oxidative stress and apoptosis in the target brain region.

2. Methods

2.1. Animals

The female Sprague-Dawley rats weighing 250–300 g were obtained from Orient Bio (Seongnam, Korea). The rats were retained in a limited access rodent facility with up to two rats per polycarbonate cage. The female rats were allowed to acclimate for seven days, housed as a pair in a controlled environment, and maintained on a 12 hours light/dark cycle during all experimental period. Water and food were provided *ad libitum*. In addition, temperature and humidity were kept at 20–23°C and 45–55%, respectively. On experiment days, acupuncture treatment and behavior experiments were administered in a silent room to minimize stress to the rats. The experimental protocols for animal usage were reviewed and approved by the Institutional Animal Care and Use Committee of Korea Institute of Oriental Medicine with reference number #17-009 (Daejeon, Korea).

2.2. Chemicals or antibodies

OVX rat injected E2 (Tocris Bioscience, Bristol, UK; catalog no. 2824), Estradiol ELISA kit (Biovision, Milpitas, CA, USA; catalog no. K7417-100). The primary antibodies used for western blot and immunofluorescence staining were 8-OHDG antibody (Abcam, Cambridge, UK; catalog no. ab48508), BiP antibody (Thermo Fisher Scientific, Lafayette, Colorado; catalog no. PA1-014A), Sigma receptor 1 antibody (Santa Cruz, California, USA; catalog no. sc-137075), pJNK antibody (Cell Signaling Technology, Boston, Massachusetts, USA; catalog no. 4668), JNK antibody (Cell Signaling Technology; catalog no. 9252), PDI antibody (Cell Signaling Technology; catalog no.3501), Ero-1α antibody (Cell Signaling Technology; catalog no.3264), Calnexin antibody (Cell Signaling Technology; catalog no.2679) and β-actin (Cell Signaling Technology; catalog no. 8457). The secondary antibodies used for western blot and immunofluorescence staining were the anti-rabbit lgG, HRP linked antibody (Cell Signaling Technology; catalog no. 7074), anti-mouse lgG, HRP linked antibody (Cell Signaling Technology; catalog no. 7076), Goat anti-Mouse IgG (H + L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor Plus 647 (Thermo Fisher Scientific; catalog no. A32728) and Goat anti-rabbit IgG (H + L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor Plus 488 (Thermo Fisher Scientific; catalog no. A-11029).

2.3. OVX surgery

The ovariectomized surgery performed under inhalation anesthesia, which was initiated in an induction chamber using a 4% isoflurane and 96% oxygen mixture and maintained with the mixture of 2% isoflurane and 98% oxygen at a flow rate of 0.8 L/min. An ovaries remove, ovariectomy, was performed through abdominal incision. Two weeks after ovariectomy, we measure the serum E2 levels and observing mature exfoliated epithelial cells in vaginal smears for confirmed the success of the surgery in the OVX rats (data not shown). After we confirmed the absence of estrus, we applied acupuncture stimulation to the acupuncture point at SP6 or E2 injection. The E2 injection group was designed based on the research of Pinceti in 2016.[24] The E2 (2.5 μg/kg, i.p.) dissolved in corn oil once/day for 3 days. Their groups estrogen levels differences are shown in Fig. 1C.

2.4. Mechanical acupuncture instrument (MAI) stimulation and acupuncture point

This study investigated the effect of SP6 point. The acupuncture stimulation at the SP6 point improves women's general health. Recent studies have shown that acupuncture at SP6 significantly increased estradiol and significantly decreased follicle-stimulating hormone (FSH) and that these hormonal changes were accompanied by a substantial decrease in the severity of hot flashes in premenopausal women that had ovariectomies.[25–27] It is an important point in the treatment of digestive, gynecological and emotional conditions. SP6 was selected as an effective locus for depression due to female hormone deficiency.

Traditional manual acupuncture is the act of inserting a needle into the acupuncture point, which sometimes involves twisting the needle. The MAI was developed to mimic the vibrations produced by manual acupuncture stimulation. The MAI was gained from Daegu Haany University (Daegu, Korea). The acupuncture needles (0.3 × 30 mm, DongBang Medical, Gyeongi-do, Korea) were inserted bilaterally 3 mm deep at SP6 or non-acupoint. Rats were anesthetized using isoflurane, and the needles were inserted into acupuncture points, vibrated with the MAI for 30 seconds, maintained up to 1 minute after needle insertion and subsequently withdrawn.[28] The MAI stimulation was applied once daily for 4

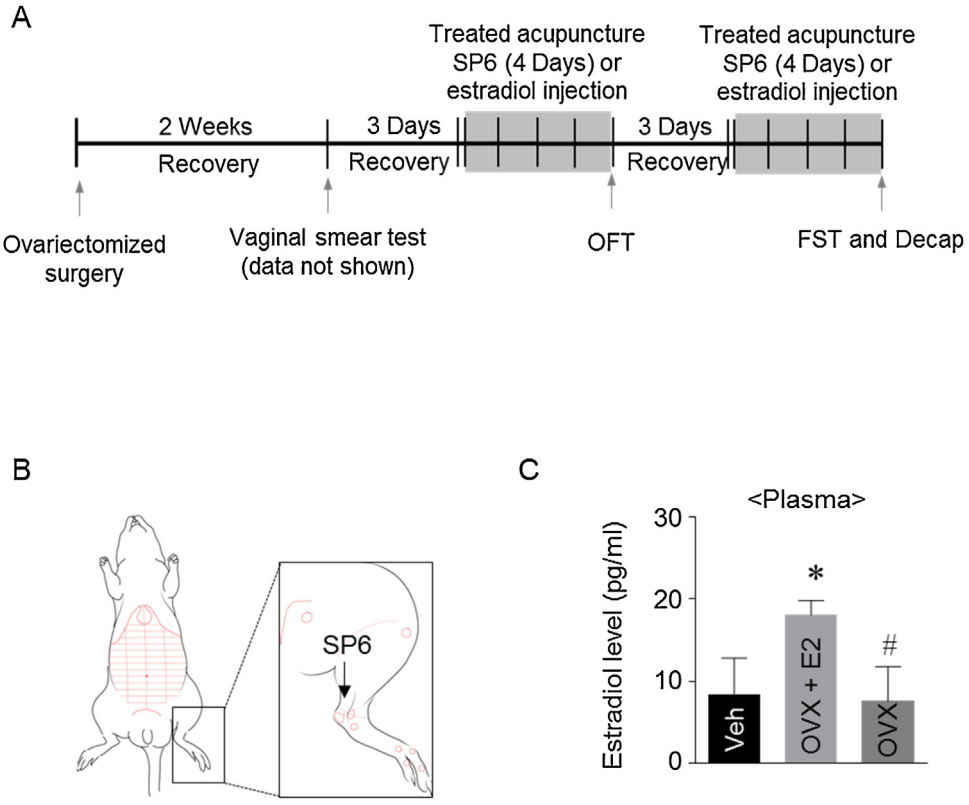

Fig. 1. Schematic diagram and map of acupuncture point SP6. Schematic diagram showing the stimulation of acupuncture of the rats. (A) Acupuncture stimulation was performed at the acupuncture point Sanyinjiao (SP6). (B) The release of E2 in the plasma (2.5 μg/kg per day, $n = 4$ for each group). (C) The data were analyzed using repeated measures unpaired t-test ($t = 3.976$, $p = 0.0165$).

consecutive days. The depth of acupuncture needle insertion at SP6 or the non-acupuncture point (located at the upper part of the left buttock) was controlled based on the trans positional method, which locates animal acupuncture points on the surface of their skin corresponding to the anatomic site of human acupuncture points (Fig. 1B).[29]

2.5. Experimental groups

Experiments were designed to consider the effects of acupuncture stimulation on OVX-induced depression-like behavior and associated protein expression.

The rats were divided into four groups and subjected to the following treatments: OVX with estradiol injection (OVX + E2), OVX alone (OVX), OVX with bilateral acupuncture at the SP6 (OVX + SP6) and OVX with a non-acupoint at the upper part of left buttock (OVX + NonAcu).

2.6. Enzyme-linked immunosorbent assay (ELISA)

An ELISA kit was used to measure the levels of the plasma E2 in the OVX, OVX + Veh and OVX + E2 rat groups. The detailed steps were carried out according to the manufacturer's protocol. Very briefly, Add 50 μL per well of the six rat Estradiol standard solutions in the pre-coated 96-well plate. And then, 50 μL sample diluent buffer into the sample control well. Add 0.35 mL of prepared working wash solution into each well. Wash plate 5 times, each time leave washing buffer in the wells for 2 minutes. And 50 μL of HRP-conjugate reagent into each well (except the Zero well) and incubate plate at 37°C in dark for 30 minute. Add 50 μL of Chromogen solution A and 50 μL of Chromogen solution B into each well. Incubate plate at 37°C in dark for 15 minutes. After 15 minutes of incubation, the stop solution into each well and color development is stopped and the absorbance of the tubes is read at 450 nm.

2.7. Behavioral assessments

2.7.1. Forced swimming test (FST)

The OVX rats were located in a cylindrical tank (38 cm width × 60 cm height), which was filled with water (24 ± 1°C) to a depth of 40 cm to avoid the rats from supporting themselves by touching the bottom with their tail. The forced swim test proceeded for 5 minutes, the rats were removed from the cylinders. The water in the cylinder was replaced after every trial. This experiment used a time-sampling technique to score numerous types of behavior. The scoring was conducted by a blinded experimenter which determined the immobility time using a SMART v3.0 tracking system (Panlab, Barcelona, Spain).

2.7.2. Open-field test (OFT)

An OFT is usually performed to measure locomotor activity in rodents.[30] Briefly, the apparatus used in the present study consisted of a gray square 120 cm × 120 cm × 40 cm. Rats were placed in the center, and total travel distance was determined manually for 5 minutes. After each test, the apparatus was cleaned. The images were detected on a computer with a SMART v3.0 tracking system, and the distance traveled and time spent in the central square (60 × 60 cm) were calculated automatically. The total traveled distance in the zone was manually recorded.

2.8. Western immunoblotting

The rats underwent inhalation anesthesia with 4% isoflurane and were sacrificed 20 minutes after the final acupuncture

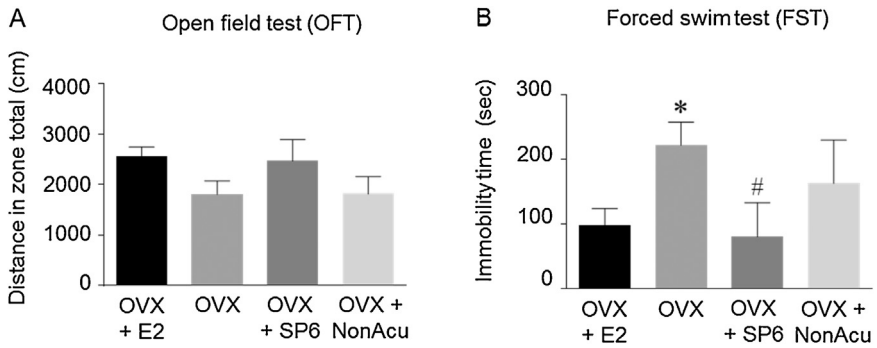

Fig. 2. Effects of acupuncture stimulation on depression-like behaviors. Quantification of total distance traveled in the OFT ($n = 8$ for each group) and immobility time during the FST ($n = 7–8$ for each group) (A, B). The data were analyzed using repeated measures ANOVA followed by Tukey test. $^{*}p < 0.05$ vs. OVX + E2 group; $^{\#}p < 0.05$ vs. OVX group. Values are expressed as the mean ± SEM.

treatment. The brains were removed, thick brain slices were serially cut in a rodent brain matrix, and the amygdala was removed. The amygdala samples were lysis with RIPA buffer (50mM NaCl, 50mM Tris pH 7.4, 0.5% DOC, 0.1% SDS, 1% NP-40, 1mM EGTA, 1mM Na_3VO_4, 1mM PMSF, 1mM Na-F, 1 g/mL aprotinin, 1 g/mL leupeptin). The samples were then sonicated for 30 seconds on ice and incubated for 1 hour 30 minutes at 4°C. The samples were centrifuged again at 13,000 rpm for 30 minutes at 4°C to obtain samples free of large debris. Fractionated proteins were used for 4–15% gradient sodium dodecyl sulfate–polyacrylamide gel (Bio-Rad Laboratories, Hercules, CA, USA) electrophoresis, and divided proteins were transferred to a nitrocellulose membrane. The concentration of solublized proteins in the supernatant was determined based on the Bradford method using the Bio-Rad Protein Assay (Bio-Rad Laboratories). The membrane was blocked with blocking buffer containing 5% skim milk in a combination of Tris-buffered saline and Tween-20 (TBST) and then probed with primary antiserum. The BiP antiserum was diluted 1:2000 and Sigma receptor 1 antiserum was diluted 1:1000. The pJNK, JNK, CHOP, PDI, Ero1-Iα and Calnexin were diluted 1:1000 and β-actin was diluted 1:2000. All membranes were incubated overnight at 4°C. The next day, after 3 washes with TBST for 10 minutes, the membrane was incubated with the appropriate secondary antiserum at a dilution of 1:1000 or 1:2000 for 2 hours at room temperature. Membranes containing immunoreactive proteins were developed using enhanced chemiluminescence reagents (Thermo Fisher Scientific). Protein bands were detected and analyzed using a FusionSL4-imaging system, and quantification of the immunoblotting bands was achieved with ImageJ.

2.9. Immunofluorescence staining

Immunofluorescence staining was used to examine BiP and 8-OHDG expression. After perfusion, the brain tissue was fixed in 10% neutral buffered formalin (Sigma), embedded in paraffin and cut into 5 μm sections. Next, the samples were deparaffinized, hydrated and stained by standard methods. For immunofluorescence analysis, brain sections were de-paraffinized. Antigen retrieval was performed by heating in 10mM sodium citrate buffer (pH 6.0) for 10 minutes using a microwave. Specimens were blocked in 5% blocking solution for 1 hour at room temperature followed by incubation with primary antibody at 4°C overnight. The BiP and 8-OHDG antibody were diluted 1:1000. The secondary antibodies used here were Alexa Fluor 647 or Alexa Fluor 488 antibodies corresponding to the species of primary antibody (1:1000). Coverslips were then dried and mounted onto slides using permount. The microscopy was performed using a fluorescence microscope (BX51; Olympus, Hamburg, Germany). The images were recorded and then processed using Olympus CellSense software (version 1.41).

2.10. Statistics

Differences in the number of immunoreactive pixels per measured area and various behaviors between groups performed in this study were determined by one-way ANOVA or *t*-test followed by Tukey honestly significant difference test using GraphPad Prism 6 (GraphPad Software Incorporation, San Diego, CA, USA). Data are expressed as the mean ± SEM for each group ($n = 4–5$ per group). A p value <0.05 was considered statistically significant.

3. Results

3.1. MAI stimulation of the acupuncture point SP6 in OVX reduced depression-like behaviors in the FST

This study was performed to determine whether MAI stimulation alterations depression-like behavior in OVX rats. The OVX rats were treated with MAI for 4 days, and OFT and FST were measured 20 minutes after the last MAI stimulation (Fig. 1A). The plasma E2 levels in the OVX + E2 group were significantly higher than those of the OVX group and OVX + Veh ($F(2, 6) = 7.415$, $p = 0.0239$). So, OVX + E2 groups were used as controls for OVX group (Fig. 1C). The total distance in the OFT tended to increase by stimulation of acupuncture point SP6, but there was no significant difference (Fig. 2A). The OVX rat group showed significantly increased immobility time compared to the OVX + E2 group, and the OVX + SP6 ($F_{(3, 27)} = 9.153$, $p = 0.0002$) group, but not the OVX + NonAcu group, recovered the immobility time (Fig. 2B).

3.2. MAI stimulation of acupuncture point SP6 in OVX-induced expression of 8-OHDG and BiP in the amygdala

To determine the affected brain regions, we performed immunofluorescence analysis of each group (Fig. 3A). Compared to the OVX + E2 group, the OVX groups showed significantly increased protein expression of 8-OHDG in the amygdala. The stimulation of acupuncture point SP6 significantly decreased the protein expression of 8-OHDG compared to that of the OVX groups ($F_{(3, 21)} = 17.00$, $p < 0.0001$) (Fig. 3B). Compared to the E2 group, BiP protein expression also significantly increased in the amygdala of the rats in OVX group. The stimulation of SP6 significantly decreased the increased BiP expression in the OVX group. ($F_{(3, 11)} = 15.47$, $p = 0.0003$) (Fig. 3C).

3.3. MAI stimulation of acupuncture point SP6 in OVX reduced ER stress and oxidative stress levels in the amygdala

The estradiol deficiency increases the ROS generation, which activates ER stress and oxidative stress response. To determine the

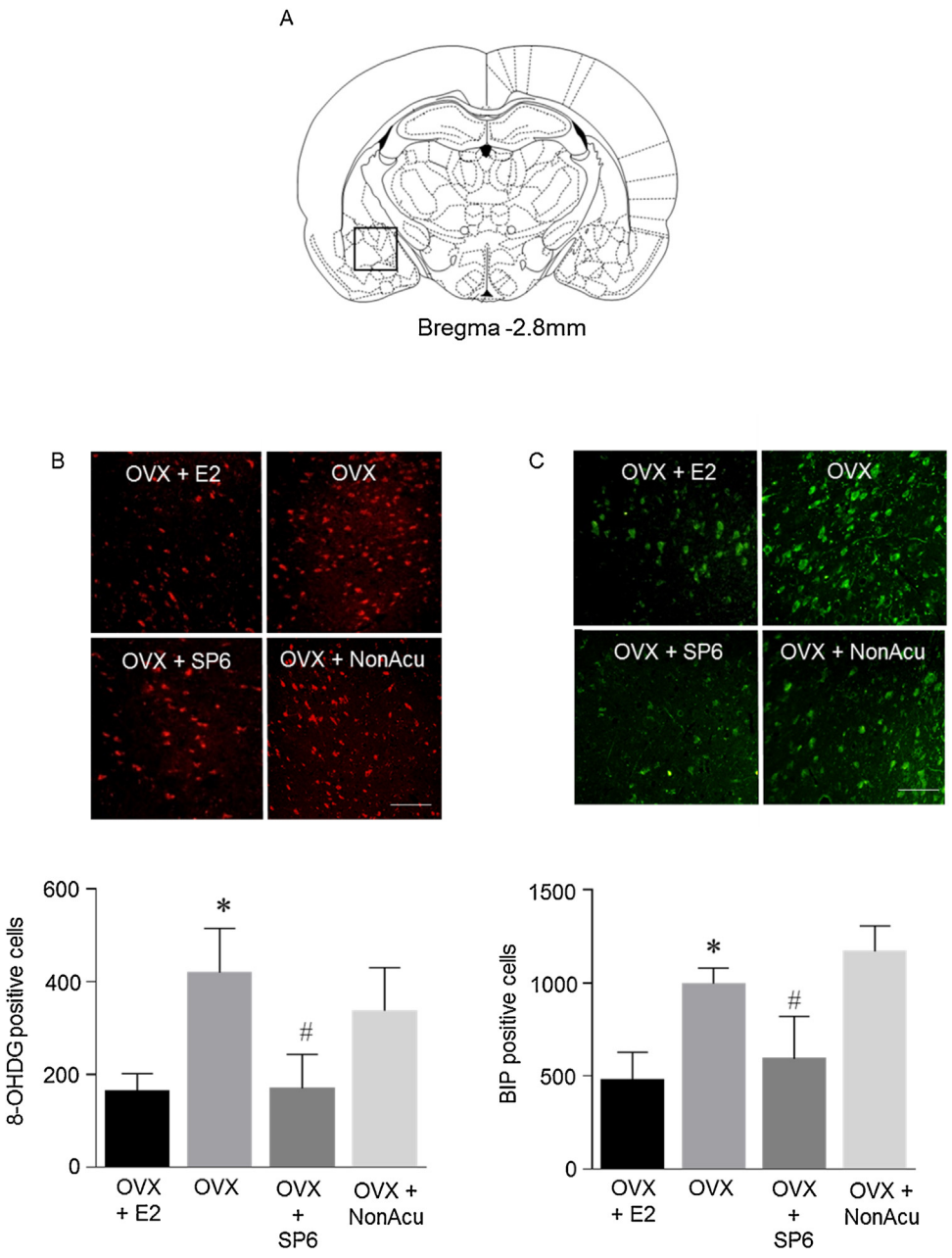

Fig. 3. Effects of acupuncture stimulation on 8-OHDG and BiP levels in the amygdala. The schematic coronal diagram from a rat brain atlas (Palkovits, Miklos, 1983) was superimposed on a photomicrograph of a representative coronal brain section showing the location of the active membrane in the CeA (A). The release of 8-OHDG in the CeA ($n = 6–8$ for each group). The release of mature BiP in the CeA ($n = 4–5$ for each group) (B, C) Representative micrographs showing the expression of 8-OHDG and BiP in the CeA. The scale bar represents 100 μm. The results are presented as the positive cell number of 8-OHDG and BiP immunoreactive cells. The mean 8-OHDG and BiP levels are expressed as a percentage of the control. The data were analyzed using repeated measures ANOVA followed by Tukey test. $^{*}p < 0.05$ vs. OVX + E2 group; $^{\#}p < 0.05$ vs. OVX group. Values are expressed as the mean ± SEM.

anti-depressive effect of MAI stimulation of acupuncture point SP6, we measured the ER stress and oxidative stress levels in the amygdala. The results indicated the expression levels of ER stress makers and oxidative stress markers. In detail, the 8-OHDG, pJNK and Sigma receptor 1 are used as makers of oxidative stress. And the markers of ER stress are BiP, Ero1-Iα, PDI, Calnexin and Sigma receptor 1. The increased BiP expression in OVX rats was significantly decreased after MAI stimulation of acupuncture point SP6 ($F_{(3,\ 9)} = 6.215$, $p = 0.0142$) (Fig. 4A). The increased Sigma receptor 1 levels in the MAI stimulation of acupuncture point SP6 and NonAcu group were not significantly decreased compared to those of the OVX rats (Fig. 4B). The pJNK expression in the amygdala was significantly decreased compared to that in the OVX rats following MAI stimulation of acupuncture point SP6 ($F_{(3,\ 8)} = 16.16$, $p = 0.0009$) (Fig. 4C). In addition, the MAI stimulation of acupuncture point SP6 group showed significantly decreased PDI expression compared to the OVX rat group ($F_{(3,\ 9)} = 16.91$, $p < 0.0005$) (Fig. 4D). Stimulation of acupuncture point SP6 rats showed significantly decreased Ero1-Iα compared to the OVX rat group ($F_{(3,\ 10)} = 19.60$, $p = 0.0002$) (Fig. 4E). The increased Calnexin level of OVX rats was significantly decreased by MAI stimulation of acupuncture point at SP6 ($F_{(3,\ 10)} = 10.89$, $p = 0.0017$) (Fig. 4F). These results are consistent with the immunohistochemical results and may potentially explain the antidepressant effects of MAI stimulation of acupuncture point SP6.

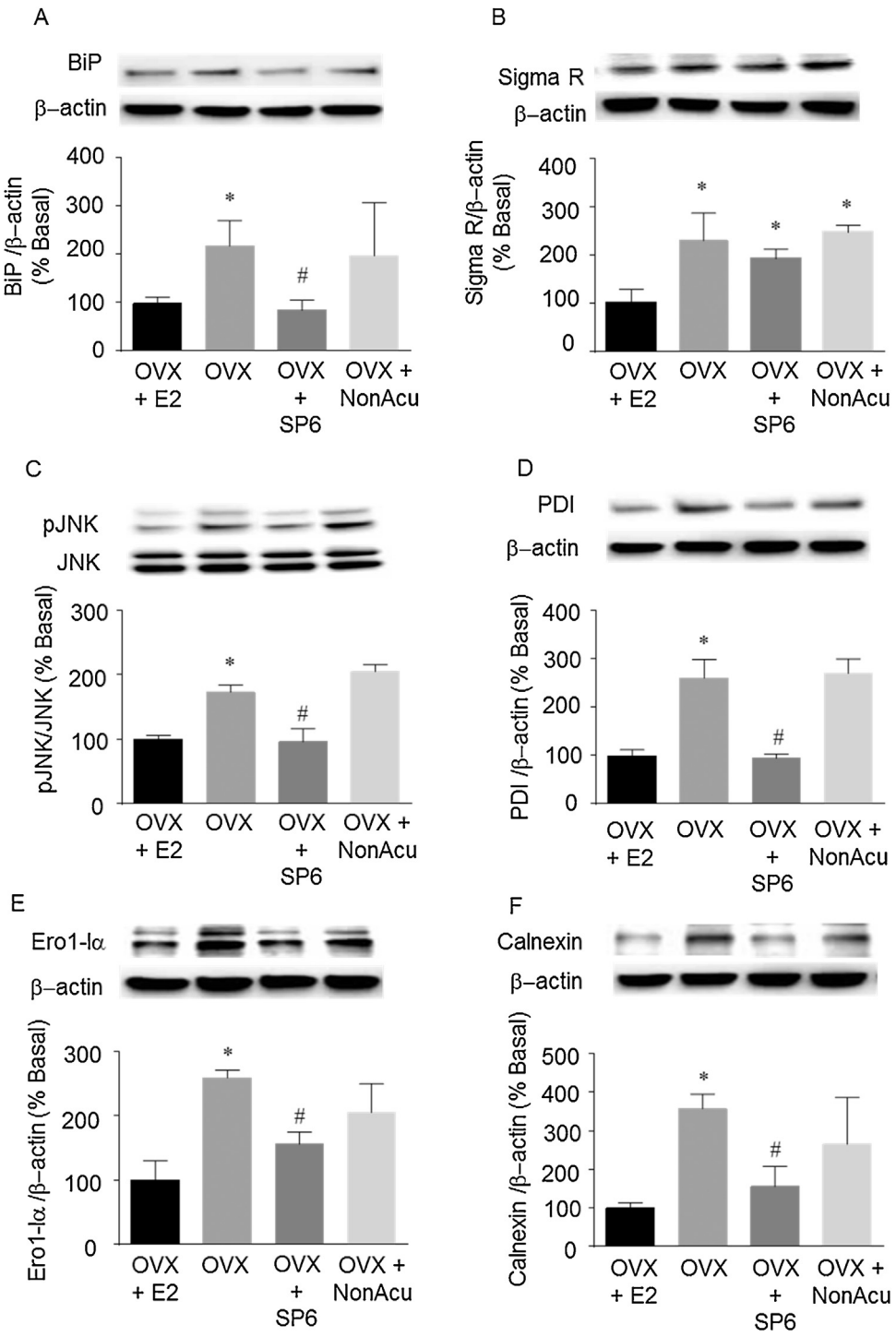

Fig. 4. Effects of acupuncture stimulation on ER stress and oxidative stress protein expression in the amygdala. The expression of BiP, Sigma receptor 1, pJNK, PDI, Ero1-lα and Calnexin in the CeA ($n = 3–4$ for each group). The data were analyzed using repeated measures ANOVA followed by Tukey test. $^{*}p < 0.05$ vs. OVX + E2 group; $^{\#}p < 0.05$ vs. OVX group. Values are expressed as the mean ± SEM.

4. Discussion

In East Asian nations, acupuncture has been extensively used to treat various mental illnesses, such as stress, anxiety and depression. Acupuncture can regulate of the brain functional system by direct activation of neurotransmitters. Thus, it contributes to the biochemical balance in the central nervous system by regulating neurotransmitters that control health and disorder. Many studies have the effects of acupuncture on depression at the molecular level. SP6 is one of the most generally used acupuncture points in acupuncture treatment to alleviate psychic and psychosomatic dysfunction, such as depressive symptoms and anxiety disorders.[31,32] Our results also demonstrated that stimulation of acupuncture point SP6 reduced OVX-induced depression-like behavior via alleviation of the ER stress and oxidative stress in the amygdala.

The present study established that stimulation of the SP6 acupuncture point using a MAI significantly decreased the increased immobility time in the FST induced by OVX. The MAI

has the benefit of a shorter stimulus time (30 s) than that of typical electroacupuncture, which is nearly 30 minutes, and involves minimal handling and restraint to decrease stressful behaviors. Interestingly, even a brief 30 seconds acupuncture reduced behavioral response. In addition, stimulation of acupuncture point SP6 resulted in significant differences compared to a non-acupoint, as shown through that the stimulation of SP6 has a specific alleviatory effect on OVX-induced behavior. According to our results, the acupuncture point SP6 stimulation has an anti-depressive effect.

Next, we demonstrated that MAI stimulation of SP6 significantly decreased the 8-OHDG and BiP expression in the central nucleus of the amygdala. The amygdala is significant for the interpretation of emotional meaning, and it has been reported that patients with depression have impaired role during emotional tasks.[33–37] For this reason, the molecular mechanisms in amygdala are an important topic of depression research. Our results show that in depression due to estrogen deficiency, ER stress and oxidative stress were increased in the amygdala. When depressive behavior was reduced by repeated stimulation of acupuncture point SP6, ER stress and oxidative stress were also decreased. These results can explain how stimulation of acupuncture point SP6 contributes to the mitigation of homeostasis imbalance by hormone deficiency.

Using Western blot analysis, we found that stimulation of acupuncture point SP6 decreased the increased BiP expression in the amygdala by OVX. This result can explain how stimulation of acupuncture point SP6 alleviates ER stress induced by estrogen deficiency. BiP is an ER stress maker that is important in estrogenic stimulation in different tissues, such as mammary brain and lands. The estrogen receptors dysfunction increased expression of BiP, NF-κB and caspase-3, thereby inducing cell stress.[38–42] Both BiP and estrogens have been associated with cell death regulation, cell survival, proliferation and metastasis.[43–45] Our results demonstrated that ER stress caused by hormonal imbalance is mitigated by stimulation of acupuncture point SP6.

The c-Jun N-terminal kinase (JNK) is activated in response to extensive range of cellular stresses as well as in response to cell apoptosis and inflammatory mediators. The JNKs control various processes, such as brain development, memory formation and repair, but they are also potent effectors of neuroinflammation, apoptotic activities and neuronal death. Treatment with E2 inhibited pJNK in a spinal cord injury rat model.[46] Pretreatment with E2 significantly maintained neural cell viability, reduced apoptosis, and inhibited pJNK.[47] Our results indicated that stimulation of acupuncture point SP6 decreased the increased pJNK expression by E2 deficit. This result supports the hypothesis that stimulation of acupuncture point SP6 alleviates the brain imbalance caused by hormone deficiency through phosphorylation of JNK.

The protein disulfide isomerase (PDI) is a candidate pathway for coupling ER stress to oxidant generation. During the course of cerebral ischemia and neurodegenerative diseases, PDI induces accumulation of denatured proteins and toxicity to neurons.[48] Under normal circumstances, PDI up-regulation would decrease the abnormal accumulation of misfolded proteins to protect neurons. Despite the neuronal protection function of PDI, there is little research into changes of PDI during depression. According to the Fig. 4D, that PDI is increased to restore estrogen deficit, and decreased PDI was restored by acupuncture stimulation.

The endoplasmic reticulum oxidoreductin-1 (Ero1) is an oxidoreductase enzyme that induces apoptosis that releases calcium into the Ero1 is an oxidoreductase that communicates disulfide bonds to PDI, which helps conformation structure of ER protein.[49] Some ER stress studies suggested that Ero1-Iα is responsible for inositol 1,4,5-triphosphate (IP3) receptor-induced Ca2 + release and initiating apoptosis. Thus, Ero1-Iα activation can explain the misfolded proteins and ER stress level in the OVX rat model. Our results indicated that stimulation of SP6 decreased the Ero1-Iα expression by OVX. This result supports the hypothesis that the stimulation of acupuncture point SP6 reduced ER stress and oxidative stress by estradiol deficit depression.

Several molecular chaperones, such as Calnexin and calreticulin, play crucial roles in the folding of freshly created proteins and quality control pathways in the ER.[51,52] Calnexin is one of the key regulators for ion channel role and serves as a therapeutic target for treating signs associated with channe-lopathies, such as behavioral anomalies. Our results indicated that stimulation of acupuncture point of SP6 decreased the Calnexin expression by OVX.

Taken together, these results show that estradiol hormone deficiency by OVX surgery induces brain imbalances and promotes protein misfolding. In this process, various protein was increase such as misfolded protein and ER-stress related protein. However, stimulation of acupuncture can be expected to moderate the brain dysfunction due to estradiol deficiency. Imbalances of the brain were returned to normal levels, indicating that various ER stresses and oxidative stresses were reduced. It is not known how acupuncture relieves the dysfunctional brain balance due to hormone deficiency, but there are some predicted mechanisms. Activation of MEK/ERK plays an important role in regulatory cell survival by resisting ER stress-induced cell death signaling.[53] In acupuncture studies, there has been active research on the change in ERK by stimulation of acupuncture points. Acupuncture stimulation of PC6 increased the phosphorylated ERK1/2 and the phosphorylated CREB in the hippocampus and prefrontal cortex in the chronic unpredictable mild stress rat model.[54] Some research has shown that acupuncture could recover hippocampal function by modulating the cAMP/PKA/CREB signaling pathway, which represents a molecular mechanism of acupuncture for cognitive function in cerebral multi-infarction rats.[55] Our results also suggest that the MAPK pathway may act to reduce ER stress and oxidative stress. The recovery of pJNK, a MAPK protein, by acupuncture is direct evidence that acupuncture relieves ER stress, oxidative stress and apoptosis.

Through these mechanisms, acupuncture can be an effective treatment for depression due to hormone deficiency. This can be used as a basis for future use of acupuncture in the treatment of menopausal depression. Our results thus far will provide a scientific basis for SP6 acupuncture treatment in clinical practice.

Conflict of interest

The authors declare no conflict of interest.

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Ethical statement

The experimental protocols for animal usage were reviewed and approved by the Institutional Animal Care and Use Committee of Korea Institute of Oriental Medicine with reference number #17-009 (Daejeon, Korea).

Data availability

Data will be made available upon request.

Author contributions

S.Y. Seo and J.Y. Moon performed the experiments and wrote the manuscript. O.S. Kwon, S.K. Bang, S.P.Kim and K.H. Choi helped with the interpretation of the results. S.Y. Kang and Y.H. Ryu contributed to project planning and manuscript writing, and supervised all the work. All authors discussed the results and contributed to the final manuscript.

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